Analysis of Germline and Somatic *PTEN* Profiles and their Role in the Racial Disparity in Endometrial Cancer Outcome

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ABSTRACT

Endometrial cancer is the most common gynecological malignancy in the United States. Poorer prognosis and higher mortality rates for endometrial cancers in African-Americans compared to those in whites have revealed a racial disparity in this disease. In contrast to other solid tumors, several studies paradoxically have supported somatic *PTEN* mutations and microsatellite instability (MSI) as favorable prognostic features. The aim of this study was to determine whether there is a racial disparity in the frequency of these molecular alterations that may contribute to differences in endometrial cancer outcome.

We scanned paired germline and tumor DNA from 53 endometrial cancer patients for somatic mutations in the *PTEN* gene, loss of heterozygosity (LOH) around 10q22-q24, and global MSI according to Bethesda recommendations. Fourteen somatic mutations were found in 8 of 26 (31%) tumors from African-American (AA) individuals and were almost exclusively a feature of endometrioid cases (11 of 14 mutations; 79%). Eleven somatic *PTEN* mutations were found in 7 of 27 tumors (26%) from white women (NAA), with 5/11 of the mutations (45%) present in endometrioid cases. Of the 25 informative AA samples, 15 (60%) exhibited LOH at one or more loci, while LOH was observed for at least one locus in 19 of 26 (73%) informative NAA samples. Consistent with previous studies, 5/25 (20%) AA and 5/26 (19%) NAA patients were MSI-positive.

In addition, 10 single nucleotide polymorphisms (SNPs) and their reconstructed haplotypes were examined as host factors to investigate the association of specific genomic regions of the *PTEN* locus with endometrial cancer and its prognosis. Most importantly, the distribution of haplotypes revealed significant differences between the

two racial groups (χ^2= 13.814, P = 0.032). In particular, haplotype 3, CTIAGTATCC, was most clearly over-represented in AA (37%) compared to NAA (15%, P = 0.014).

The results of this study confirmed previous evidence that somatic *PTEN* mutations are associated with the favorable endometrioid histology (P = 0.015), irrespective of race. Additionally, novel differences were observed between the two racial groups in the *PTEN* haplotype analysis. Although no specific associations were found in this limited haplotype analysis, haplotype 3 was identified as a candidate for further study as a germline component in endometrial cancers that perhaps may predispose AA to certain unfavorable prognostic factors and thus contribute to the racial differences in outcome. A trend among the haplotype distribution was also observed between the endometrioid and non-endometrioid study groups (χ^2= 11.870, P = 0.065), further suggesting that certain haplotypes perhaps could affect histology and consequently influence the racial disparity. Taken together, we conclude that none of the somatic molecular alterations per se independently account for the observed racial disparity in endometrial cancer outcome; however, cumulatively, these data suggest that genetic factors likely contribute to this disparate clinical outcome, perhaps additively or acting in concert, and certainly require further investigation.

INTRODUCTION

Endometrial cancer is the most common type of uterine cancer as well as the most common cancer of the female reproductive system in the United States, accounting for 6% of all women's cancers (1). While the mortality rate among white women has decreased over the past 20 years, it has remained constant among other racial groups. In particular, the mortality rate of African-American women with endometrial cancer is nearly two-fold higher than that of whites despite a slightly lower incidence. From 2000-2003, the National Cancer Institute reported age-adjusted endometrial cancer incidence of 19.5/100,000 African-American women and 23.3/100,000 white women; however, the mortality rates for these years were 7.1/100,000 African-American women with endometrial cancer and only 3.9/100,000 white women with the same disease (2). Moreover, in their 1996-2002 review, the five-year relative survival was only 60% in African-American patients with endometrial cancer compared to 85% in white women with this disease. Together, these data strongly suggest a disparity between race and endometrial cancer outcome.

The basis for this disparity is currently being investigated. While progress has been made towards elucidating the epidemiology and natural history involved, its mechanism remains largely unknown. One factor which has been shown to be associated with the less favorable prognosis observed in African-American women is the difference in usage of estrogen replacement therapy, which is more often prescribed to white patients (3, 4, 5). While use of this hormone in an unopposed manner increases the risk of endometrial cancer, estrogen-induced cancers are usually well differentiated; thus, estrogen replacement therapy is associated with favorable tumor grade and contributes to

better survival in whites. Progestational agents, however, can be added to decrease endometrial proliferation and increase *PTEN* expression, which is why most hormone replacement therapies use a combination of estrogen and progesterone (6, 7). The poor outcome in African-Americans with endometrial cancer can also be attributed to the higher frequency of unfavorable prognostic features. African-Americans are more often diagnosed with poorly differentiated, deeply invasive, aggressive, and advanced stage tumors (8-14). Data demonstrating that African-Americans tend to receive delayed diagnosis and less aggressive treatment suggest that providing these patients with improved health care could improve their endometrial cancer survival and might partially explain their more frequent presentation at an advanced stage (11, 12, 13). African-Americans have a higher incidence of more aggressive type II tumors, including serous, mixed epithelial, and mixed mullerian tumors, rather than less aggressive type I endometrioid carcinomas, which occur more frequently in whites (8-14). The etiology of type II carcinomas differs from that of the most common type I endometrioid carcinomas (15, 16). Hence, racial disparity in outcome may reflect differences in etiologic exposure, genetics, or other factors that lead to higher rates of aggressive tumors (17).

Recent studies have offered several genetic explanations for the racial disparity in endometrial cancer. The p53 protein regulates cell-cycle inhibition and apoptosis in response to DNA damage; thus, mutation or deletion of *TP53* resulting in loss-of-function often leads to uncontrolled cell growth (18, 19). Contrary to the rapidly degraded p53 in normal cells, the altered protein resists degradation and accumulates in the nucleus, where it can be detected by immunohistochemistry as p53 overexpression (18, 20). Studies have shown that p53 overexpression occurs more frequently in African-

American women and is an independent variable associated with poor survival as well as a potential predictor of recurrence (21-24). The p27 protein, a cyclin-dependent kinase inhibitor also involved in regulating the cell cycle, is thought to play a similar role in endometrial carcinogenesis, but its role has been less widely studied (25).

The phosphatase and tensin homolog gene (*PTEN*), located at 10q23.3, is a ubiquitously expressed tumor suppressor phosphatase that mediates cell cycle arrest and apoptosis (26, 27). It is involved in the pathogenesis of many types of sporadic solid tumors. Germline *PTEN* mutations have been found in 85% of classic Cowden Syndrome, a heritable, multiple hamartomatous tumor syndrome associated with high risk of breast, thyroid, and endometrial carcinomas (27, 28, 29, 30). Somatic mutations and/or deletions of *PTEN* as well as non-genetic *PTEN* inactivation have been shown to play a major role in the initiation of endometrial cancer, suggesting that these events occur early in endometrial carcinogenesis, prior to the loss of normal differentiation or histology (31, 32). As the tumors transition from premalignant to malignant disease, additional *PTEN* damage accumulates. Thus, immuohistochemical methods can be used to detect loss of PTEN expression and used as an informative biomarker for endometrial neoplasia, including precancerous lesions. *PTEN* is mutated in ~30-40% of endometrial cancers (31, 33). Previous studies have found that somatic mutations in *PTEN* are four-fold more frequent in tumors from whites relative to those from African-Americans and are associated with favorable survival in advanced endometrial cancer (34, 35). Thus, *PTEN* and the chromosome region in which it resides are excellent candidates for the genetic analysis of endometrial cancer as it relates to racial disparity in presentation and outcome.

In another heritable cancer syndrome, hereditary non-polyposis colorectal cancer, endometrial carcinoma is the most common extra-colonic cancer, exceeding the incidence of colorectal cancer in women carriers (36, 37). In this syndrome, germline mutations of DNA mismatch repair genes often lead to microsatellite instability (MSI) (38, 39). Occurring in ~20% of cases with endometrial cancer, MSI has been associated with endometrioid histology and thus identified as a favorable prognostic feature for endometrial cancer (34, 38). Taken together, both somatic *PTEN* mutation and MSI status constitute somatic genetic factors that potentially play a role in endometrial cancer progression and may even contribute to the racial disparity seen in this disease.

Previous association studies on this subject have focused primarily upon individual single nucleotide polymorphisms (SNPs). Taking advantage of linkage disequilibrium (LD), haplotype-based studies have the potential to provide much more powerful results. LD involves the coinheritance of SNPs that possess strong allelic associations and results in block-like segments, or haplotypes, which are passed through generations (40). Each discrete block is bounded by regions of historical recombination and contains only a few common haplotypes. These common haplotypes often occur at varying frequencies among racial groups when populations have been separated and new mutations have not had much opportunity to spread; therefore, the distribution of haplotypes may provide clues to underlying genetic differences in AA and NAA with endometrial cancer.

In order to clarify the molecular pathogenesis of this disparity, we chose to examine the differences in various somatic and germline *PTEN* profiles along with global MSI of African-Americans compared to those of whites. This study confirmed previous

findings regarding endometrial cancer, including the association between *PTEN* mutation and the more favorable endometrioid histology, irrespective of race (34, 35). Moreover, statistically significant differences between AA and NAA were observed among the *PTEN* SNP haplotype distributions, identifying common haplotypes and trends that with further investigation may be found to play a role in the disparate outcomes of these two groups. Undoubtedly complex, this racial disparity in endometrial cancer appears to involve numerous molecular alterations in need of more detailed study.

MATERIALS AND METHODS

Patients. Fifty-three patients, consisting of 26 self-described African-Americans (AA) and 27 whites of Western European descent (NAA) with endometrial cancer, were included in the present study. The study was designed to match both racial groups for tumor histology and FIGO* stage. Each group was made up of 12 endometrioid cases (~45%), 2 mixed epithelial cases (~8%), and 7 serous cases (~26%). Additionally, 5 and 6 mixed mullerian tumor cases (~21%) were included from each respective ethnic group. The AA group consisted of 18 cases with Stage I tumors (69%), 2 cases with Stage II tumors (8%), 5 cases with Stage III tumors (19%), and 1 case with a Stage IV tumor (4%). Of the NAA, 19 were Stage I (70%), 3 were Stage II (11%), 4 were Stage III (15%), and 1 was Stage IV (4%). Age at diagnosis ranged from 36 to 87 years for all patients. Germline and tumor DNA from each patient were isolated from paraffin-embedded tissue blocks by standard techniques (41).

***PTEN* Mutation Analysis.** *PTEN* mutation analysis of all nine coding exons, exon-intron junctions, and flanking intronic sequences was performed for both germline and tumor DNA using PCR-based denaturing gradient gel electrophoresis (DGGE). For the initial PCR, each 25 µl reaction mixture contained 20 ng of template DNA, 12.5 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA), and 10mM of each primer pair. Primer sequences can be found in Table 1.1. Amplification was performed using the following protocol: 15 min initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 55°C, and 45 s at 72°C, and finally 72°C for 10 min, 98°C for 8 min, 55°C for 30 min, and 40°C for 30 min. The samples were then subjected to electrophoresis on a 10%

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polyacrylamide gel containing 5% glycerol and a linear 15-50% urea-formamide gradient at 100V for 16 hours at 60°C (42). The gels were stained using ethidium bromide and PCR products were visualized by UV transillumination. DNA from bands identified as aberrant served as the template for fluorescence-based sequencing using BigDye v3 (Applied Biosystems, Foster City, CA). Sequencing products were electrophoresed using an ABI 3730 DNA Analyzer (Applied Biosystems) and analyzed using the Sequencher software program (Gene Codes, Ann Arbor, MI) in order to determine the exact nature of each mutation/variation.

Loss of Heterozygosity Analysis. DNA from carcinoma and adjacent germline tissue was amplified using primers that define 4 highly polymorphic microsatellite markers along the *PTEN* locus (average heterozygosity > 0.75): D10S1765, AFMa086wg9, D10S541, and D10S583 (Figure 1). Primer sequences are listed in Table 1.2. Forward primers were fluorescently labeled with HEX, FAM, or TET at the 5'-position. The PCR cycling conditions were as follows: 15 min denaturation at 95°C, then 40 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were electrophoresed using an ABI 3730 DNA Analyzer (Applied Biosystems) and loss of heterozygosity (LOH) was analyzed using GeneMapper v3.5 software (Applied Biosystems). The ratio T1:T2/N1:N2 was calculated, where T and N represent tumor and normal, respectively, and 1 and 2 designate each allele. Cases in which this ratio was less than 0.67 or greater than 1.5 were considered to demonstrate LOH (42). For each sample, overall LOH was scored when one or more of the panel of four polymorphic loci showed LOH, as is standard.

Microsatellite Instability Analysis. To determine whether MSI was present, five polymorphic microsatellite markers were amplified in paired endometrial tumor and germline DNA samples using PCR. The five markers constitute part of the Bethesda Guidelines, which were developed by an international workshop hosted by the National Cancer Institute on genetic screening of HNPCC (43). Two loci were mononucleotide poly(T) and poly(A) runs (BAT25 and BAT26, respectively) and three loci contained CA dinucleotide repeats (D2S123, D5S346, and D17S250). Primer sequences for the markers are located in Table 1.3. Forward primers were 5'-labeled with HEX, FAM, or TET. Amplification was performed using 15 min denaturation at 95°C, then 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min, and a final extension for 10 min at 72°C. The PCR products were electrophoresed as previously described and the resulting data was analyzed using GeneMapper v3.5 software (Applied Biosystems, Foster City, CA).

The overall microsatellite status of each sample was determined based on the percentage of unstable loci. According to updated Bethesda recommendations, samples were defined as MSI-positive when at least 60% (3 out of 5) of the markers displayed instability (43). Only cases showing explicitly distinct additional peaks or shifts in tumor DNA in comparison to germline DNA were classified as unstable. A sample was classified as MSI-negative when it did not meet this criterion.

Linkage Disequilibrium and Haplotype Analysis. We analyzed 10 previously mapped SNPs (with a minor allele frequency $\geq$0.10) in germline DNA from all 53 patients. SNPs were spaced approximately one every 15 kb across a 147 kb region spanning the *PTEN* locus (Table 2). The amplification procedure was as follows: 15 min denaturation at 95°C, then 34 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min,

then a final extension for 10 min at 72°C. SNPs were genotyped using either fragment analysis, restriction fragment length polymorphism (RFLP), or SnaPshot primer extension methods. Primer sequences and genotyping methodologies are provided in Table 1.4. Fragment analysis and SNaPshot products were electrophoresed on an ABI 3730 DNA Analyzer (Applied Biosystems) and analyzed using GeneMapper v3.5 software (Applied Biosystems).

Following assessment of Hardy-Weinberg equilibrium (HWE) at each polymorphic locus, pairwise LD coefficients (Lewontin's *D'*) were estimated using the LDmax software program and visualized using the GOLD graphical interface (44). *D'* was calculated and plotted separately for each sample population (AA and NAA). Haplotypes consisting of the 10 SNPs were reconstructed for all samples using the PHASE v2.1 software program.

Statistical analysis. Statistical analysis was performed using Fisher's exact test. Differences were considered significant if the two-tailed *P*-value was <0.05. Haplotype frequencies were compared using a Pearson χ^2 test. Rare haplotypes with expected frequency of ≤2 in both study groups were pooled together to make the chi-square approximation accurate. A *P*-value of <0.05 was considered significant.

RESULTS

***PTEN* Mutation Analysis.** Fifty-three samples (26 AA and 27 NAA) had sufficient material for mutational analysis of the entire *PTEN* gene. Fourteen somatic mutations were found in 8 of 26 (31%) AA tumors compared to 11 somatic *PTEN* mutations identified in 7 of 27 (26%) NAA tumors (Table 3). While the frequency of *PTEN* mutations did not reveal a racial difference (P = 0.766), an association was observed with more favorable tumor histology (P = 0.015), irrespective of race. *PTEN* mutation was identified in 11/24 (46%) endometrioid tumors and only 4/29 (14%) non-endometrioid tumors (Table 4.1). Interestingly, this association was primarily attributable to the AA population as it was statistically significant in the AA group (P = 0.009), but not in the NAA group (P = 0.662). Previous studies have shown that somatic *PTEN* mutations tend to occur more frequently in MSI-positive endometrial cancers because MSI predisposes the mononucleotide repeats in exons 7 and 8, and to a lesser extent exon 5, to be hypermutable (31, 45). After testing for association between *PTEN* mutation and MSI status (P = 0.135), the favorable endometrioid histology appears to be primarily *PTEN* mutation-associated and not MSI-dependent in the present study (Table 4.2).

Loss of Heterozygosity Analysis. We analyzed 51 endometrial cancers (25 AA and 26 NAA) for which tumor and corresponding germline DNA were available using four highly polymorphic markers flanking (D10S1765, D10S541, D10S583) and within (AFMa086wg9) *PTEN* (Fig. 1). Of the 25 AA samples, all of which possessed at least one informative marker, 15 (60%) exhibited LOH at one or more loci. Among the 26 NAA samples, all of which had at least one informative marker, 19 (73%) displayed LOH at one or more loci. No significant difference in LOH status was observed in 10q22-q24

between the two racial groups ($P = 0.382$) (Table 4.1, Figure 2). Also, MSI did not occur at a higher frequency in those carcinomas that were not characterized by LOH. These observations act as a positive control on our MSI and LOH designations as the presence of MSI can often confound calling LOH status.

Microsatellite Instability Analysis. Using the Bethesda panel of five microsatellite markers (BAT25, BAT26, D2S123, D5S346, D17S250), we analyzed MSI within 51 endometrial cancers (25 AA and 26 NAA), for which tumor and corresponding germline DNA were available. Of the 25 AA samples, 5 (20%) were MSI-positive (Figure 3). Out of the 26 NAA samples, 5 (19%) were MSI-positive. While no significant difference was found regarding MSI status between AA and NAA patients ($P > 0.99$), 20% of all endometrial cancers in this study were classified as overall MSI-positive, which agrees with previous findings (Table 4.1) (34, 38).

Linkage Disequilibrium and Haplotype Analysis. We then sought to determine if host differences in and around the *PTEN* locus were responsible for the racial disparity in endometrial carcinoma. First, it is important to note that no significant departures from HWE were observed for either population (data not shown). The existence of HWE in both our patient groups serves as a quality control check on genotyping procedures, providing evidence that the sampling probably was unbiased and there probably were not problems in laboratory procedure. Also, HWE is a guide that we can use analysis procedures that are dependent on these conditions. For the AA population, moderate LD existed between SNPs 2 and 6 ($D' = 0.802$), SNPs 2 and 9 ($D' = 0.809$), and SNPs 6 and 10 ($D' = 0.832$) (SNPs are noted in Tables 1.4 and 2). In the NAA population, moderate LD was observed between SNPs 1 and 10 ($D' = 0.710$, SNPs 2 and 10 ($D' = 0.868$),

SNPs 4 and 10 (*D'* = 0.868), SNPs 6 and 10 (*D'* = 0.868), SNPs 7 and 8 (*D'* = 0.877) and SNPs 9 and 10 (*D'* = 0.759). In this same population, low LD was observed between SNPs 5 and 10 (*D'* = 0.566). Excluding these unique areas of moderate/low LD, our data show that the *PTEN* locus is primarily characterized by strong LD (*D*' = 1.00) (Figure 4). Because of this, we chose to reconstruct extended haplotypes for both patient groups using all 10 SNPs (Table 5.1). For AA, the 4 most common haplotypes capture >80% of those observed. The same 4 common haplotypes with the addition of haplotype 5 account for >80% of those observed in the NAA population. The haplotype distribution between AA and NAA revealed significant racial differences in this study (χ^2 = 13.814, *P* = 0.032) (Table 5.1). The most distinct difference was observed for haplotype 3. While 37% of AA haplotypes existed as haplotype 3, only 15% of NAA haplotypes were present in this form (*P* = 0.014). Also, there was a trend for the association between the observed haplotype distribution and tumor histology (χ^2= 11.870, *P* = 0.065). Upon further analysis, trends were identified between haplotype 3 and unfavorable tumor stage (*P* = 0.178) as well as non-endometrioid histology (*P* = 0.375). Unfavorable tumor stage was defined as FIGO stages IC or higher, in which the cancer has invaded >50% of the myometrium. In addition, a trend was observed for haplotype 3 and absence of LOH at 10q22-q24 (*P* = 0.148). Of the other common haplotypes, there was a trend for haplotype 1 to be over-represented in the AA group (35%) compared to the NAA group (24%) (*P* = 0.287). Conversely, there was a trend for haplotypes 2 and 4 to be under-represented in the AA population (6% for both) in comparison to the NAA population (17% and 15%, respectively) (*P* = 0.124 and 0.202, in that order). Interestingly, there was an association between haplotype 2 and endometrioid histology (*P* = 0.034, Table 5.2).

DISCUSSION

African-American women have a lower incidence of endometrial cancer than white women, but their disease-related mortality is significantly higher (1). Although this disparity in survival is partially attributable to the fact that African-American women are more frequently diagnosed with metastatic disease, their survival is significantly worse in both early and advanced stages (11). In cases where the time from onset of abnormal uterine bleeding to diagnosis and intensity of treatment have been the same, this disparity in outcome has been observed (46). Studies have demonstrated that aggressive tumor types account for a higher percentage of total malignancies among African-Americans compared to whites (8-14). This represents only one of the numerous unfavorable prognostic features more common among African-Americans, including poorly differentiated, deeply invasive, and advanced stage tumors. For type I and type II endometrial cancers, the developmental pathways are fundamentally different. Pure serous carcinomas typically arise directly from atrophic endometrium, rather than from endometrial hyperplasia (15, 16). Also, serous carcinomas are less strongly related to high body mass index, exogenous estrogen use, and elevated serum levels of estrogenic hormones in comparison to endometrioid cancers. Because African-Americans have a much lower incidence of endometrioid adenocarcinoma than do whites, it may be that African-Americans are less exposed to estrogens and therefore more likely to develop the aggressive tumors that arise from atrophic endometrium (47). Higher frequency of aggressive tumors may be caused by other differences in etiologic exposure and genetics. Thus, it is of interest to investigate possible racial differences in the molecular

pathogenesis of endometrial cancer that may underlie the disparity in outcome between African-Americans and whites with this disease.

A few molecular alterations have already been identified as genetic factors that likely contribute to the racial disparity in endometrial cancer outcome. The p53 protein plays an important role in cell cycle regulation and apoptosis in response to DNA damage (18, 19). Thus, *TP53* mutation often leads cells to uncontrolled growth and is involved in the pathogenesis of numerous cancers. A mutation in *TP53* can typically be detected using immunohistochemical staining because mutant p53 resists degradation and accumulates in the nucleus, whereas p53 in normal cells is quickly degraded and unable to be detected via this method (18, 20). The resulting overexpression of p53 occurs at a significantly higher frequency for African-Americans in both early and advanced stage tumors (22, 23. 24). Overexpression of mutant p53 occurs in ~20% of endometrial cancers and is associated with non-endometrioid histology, advanced stage tumors, and poor outcome. Studies have demonstrated that p53 alterations are correlated with both rapid progression and shorter overall survival (48). Moreover, evaluation of adjuvant treatment showed that p53 overexpression was associated with resistance to platinum-based chemotherapy, providing further support that dysfunctional p53 cannot mediate the apoptotic process (49). When comparing only patients with advanced stage carcinomas and mutant p53 overexpression, African-American survival is still worse than in whites, which suggests other genetic factors contribute to the racial disparity (22). In this regard, both *PTEN* mutation and MSI have been identified previously as favorable prognostic factors, although their potential role in this disparity has not been fully elucidated (34-35, 38, 50).

In the present study, we have extended this line of investigation by examining whether there are racial differences in the incidence of somatic *PTEN* mutations/deletions and MSI in endometrial cancers. Both of these molecular alterations have been associated with endometrioid histology, which is a favorable prognostic factor; however, a unique feature of our investigation was that both tumor type and stage between the two racial groups were almost completely matched. In addition to somatic genetic factors, we were also able to analyze if germline *PTEN* variation (host factors) could account for this disparity by looking at LD among 10 carefully selected SNPs in the *PTEN* region and their extended haplotypes.

The *PTEN* gene encodes a tumor suppressor phosphatase that signals down the phosphoinositol-3-kinase (PI3K)/AKT pathway and affects apoptosis and cell cycle arrest (51, 52). Somatic mutations and/or deletions of the *PTEN* gene occur in ~30-40% of endometrial cancers and also play a role in the initiation of endometrial carcinomas (31, 32, 33). These alterations have been observed in the normal-appearing endometrial gland and in endometrial pre-cancers (31, 32). In previous studies, *PTEN* mutations have been observed in whites up to four times more frequently than in African-Americans and are associated with favorable survival in advanced endometrial cancer (34, 35). Moreover, germline *PTEN* mutations are associated with Cowden Syndrome, a heritable cancer syndrome associated with an increased risk of developing endometrial cancer (28, 29, 30). To date, germline *PTEN* mutations have been identified in 85% of patients diagnosed with Cowden Syndrome.

In the present study, somatic *PTEN* mutations were detected in tumors from 31% of AA and 26% of NAA patients. No racial differences among carriers of *PTEN*

mutations were observed (P = 0.766). Evidence from other studies, however, has demonstrated that a racial difference in molecular alterations does exist (34). These studies propose that *TP53* may be a more frequent target for mutations than *PTEN* in African-Americans, resulting in higher frequencies of non-endometrioid, advanced stage endometrial cancers, whereas, *PTEN* may be a more frequent target in whites and thus endometrioid, early stage cancers predominate (34). However, our studies do not support this hypothesis most likely because we were careful to control for histology and FIGO stage across both AA and NAA. Consistent with previous studies, a correlation was observed between the presence of somatic *PTEN* mutation and endometrioid histology (P = 0.015), the latter of which is a favorable prognostic feature, irrespective of race. Out of all endometrioid carcinomas in this study, 46% exhibited somatic *PTEN* mutations compared to only 14% of non-endometrioid carcinomas. Thus, it is possible that the previously published hypothesis that higher frequency of somatic *PTEN* mutations correlates with better prognosis may be instead due to histologic difference alone. What accounts for histology, however, is currently unknown.

We were also able to verify the prevalence of LOH in the *PTEN* region among patients with endometrial cancers. LOH does not appear to contribute to the racial disparity (P = 0.382), as 60% of AA tumors and 73% of NAA tumors exhibited LOH. These data do indicate that *PTEN* is a commonly-mutated gene in endometrial cancers and is likely to exert a significant influence upon carcinogenesis (31, 33). However, because PTEN and p53 interact at several levels, the real molecular-based etiology to racial disparities in endometrial cancer outcome will not be straightforward. For example, p53 has been shown to act as a transcription factor for *PTEN* (53). Yet, *PTEN* has been

shown to autoregulate its own expression by stabilizing p53 regulation of itself in a phosphatase-independent manner (53). Paradoxically, when PTEN and p53 interact, p53 is stabilized but PTEN is degraded (55).

In order to assess global MSI, two mononucleotide repeat and three dinucleotide repeat microsatellite markers suggested by the Bethesda Guidelines were analyzed for instability. Because of their repetitive sequence, these markers have an increased susceptibility to mutation during DNA replication. Most of these mutations are normally recognized and corrected by a family of DNA mismatch repair enzymes (56). Approximately 20% of endometrial cancers, however, have mutations in multiple microsatellite sequences attributable to inactivation of the *MLH1* DNA repair gene (57, 58). Although the *MLH1* gene is generally inactivated by mutations in syndromes such as HNPCC, inactivation of *MLH1* in sporadic endometrial cancers as well as colorectal cancers is caused by methylation of the promoter region of the gene, resulting in inhibition of transcription. As expected, MSI was identified in ~20% of the AA and NAA patient groups in the present study (38, 59, 60). These data suggest that MSI in and of itself is not responsible for the observed racial disparity ($P > 0.99$), which is in agreement with previous studies. In general, MSI-positive endometrial cancers have a high frequency of inactivating frameshift mutations in genes with coding region microsatellite repeats, which likely represents the primary means through which MSI contributes to endometrial carcinogenesis (31, 45). A major genetic explanation for the association between MSI and favorable prognosis is that MSI-positive cancer cells have a nucleotide mutation frequency two to three orders of magnitude higher than that of MSI-negative cancer cells, resulting in rapid accumulation of MSI-induced genome damage above the

threshold for cell viability and subsequent activation of apoptotic pathways or increased susceptibility to antineoplastic therapeutic agents (61, 62). Thus, these cancer cells are prone to decreased editing function and/or lose their evolutionary fitness for survival before the host dies as a result of the disease. In a related hypothesis, endometrial cancers with MSI acquire a unique spectrum of somatic molecular genetic alterations. MSI-negative carcinomas typically acquire *TP53* mutation, which is associated with type II endometrial cancers and poor prognosis (35, 59, 63, 64). Much like the hypothesis regarding somatic *PTEN* mutation and favorable histology, inactivation of *PTEN* is more common in MSI-positive carcinomas and is generally associated with type I endometrial cancers as well as improved prognosis (35, 65).

In order to determine if host-related *PTEN* variation may account for the disparate prognoses, SNPs within and near the *PTEN* locus and their corresponding haplotypes were analyzed in this study. Because haplotype-based association studies are much more powerful than traditional association studies, which focus upon individual SNPs, we chose to examine the coinheritance of SNPs in tight LD and their corresponding extended haplotypes. Statistically significant differences between the two racial groups were observed in terms of haplotype distribution (χ^2= 13.814, P = 0.032). Among the four common haplotypes shared by AA and NAA, the most substantial difference was observed for haplotype 3. This haplotype was over-represented in the AA population (37%) compared to the NAA population (15%). These haplotype differences represent potential areas of interest for further study since they account for a good portion of the racial differences in haplotype distribution. We then sought to determine whether certain trends in distribution of haplotype 3 between the two racial groups may predispose AA to

non-endometrioid histology or advanced tumor stage and in this manner affect disparate outcome. While significant associations were not found for haplotype 3 in relation to tumor histology or stage, trends were observed, suggesting that in a larger study this haplotype may help influence AA to acquire unfavorable prognostic features. Moreover, the observed trend among haplotype distribution in relation to tumor histology (χ^2= 11.870, P = 0.065) provides additional support for further investigation. Host factors may interact perhaps with the environment, as hormonal milieu differences are known to exist between AA and NAA, resulting in a certain unfavorable histology that is manifest in this apparent racial disparity. Finally, as with all studies involving AAs, admixture must also be considered as this can confound SNP/haplotype analyses. Due to variation in admixture levels among AA individuals, association studies may be inadvertently subject to stratification bias (66).

Survival of African-American women with endometrial cancer is consistently worse than that of whites with the same disease despite improving health practices. The cause of this disparity is complex and undoubtedly involves behavioral and environmental risk factors, inherited susceptibility, as well as acquired molecular alterations. By increasing our understanding of specific molecular variations that may distinguish endometrial cancer carcinogenesis within AA and NAA, detection of these in pre-cancers may help us to more effectively identify patients at risk of developing endometrial cancer. Customized medical treatments may eventually be tailored to a patient's genetic profile, taking into account his or her racial ancestry, and will be capable of improving disease prevention, diagnosis, and treatment. In the present study, a racial difference was not found for mutation or LOH in the *PTEN* region or global MSI. This

study benefited from having AA and NAA series that were matched by stage and histology; however, its weakness came from its small sample size. Despite the small sample sizes, we were able to confirm certain previously published findings, including the association between *PTEN* mutation and endometrioid histology, irrespective of race, and the incidence of MSI in 20% of endometrial cancers. These observations serve as a positive control and perhaps suggest that although small, our series does reflect the larger population-at-large to a certain extent. What is novel in our study are the significant differences among *PTEN* haplotype distribution between the two groups, which suggest the potential role SNPs and their associated haplotypes may play in the study of this health disparity. Furthermore, if a haplotype is found to occur more frequently in affected individuals than unaffected individuals, there is a high probability that a genetic variant located within that region through LD is related to endometrial cancer development. Thus, we propose to perform equivalent haplotyping in AA and NAA without endometrial cancer to see if the association holds. Clearly, several pathways affect the development of endometrial cancer and a better understanding of specific genetic differences between AA and NAA is essential if we hope to decrease the disproportionate mortality rate of African-American women with endometrial cancer.

TABLES

Table 1.1 – Primer sequences for *PTEN* mutation analysis using DGGE.

***PTEN* Exon**	**Primer Sequence***
E1 F	5'-CGT CTG CCA TCT CTC TCC TCC T-3'
E1 R	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT CCG TCT ACT CCC ACG TTC T-3'
E2 F	5'-CGT CCC GCG TTT GAT TGC TGC ATA TTT CAG-3'
E2 R	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTC TAA ATG AAA ACA CAA CAT G-3'
E3 F	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTA AAT GGT ATT TGA GAT TAG-3'
E3 R	5'-GCG CGA AGA TAT TTG CAA GCA TAC A-3'
E4 F	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAC ATT ATA AAG ATT CAG GCA ATG-3'
E4 R	5'-GAC AGT AAG ATA CAG TCT ATC-3'
E5.1 F	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTT TTT TCT TAT TCT GAG GTT ATC-3'
E5.1 R	5'-TCA TTA CAC CAG TTC GTC C-3'
E5.2 F	5'-TCA TGT TGC AGC AAT TCA C-3'
E5.2 R	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAA GAG GAA AGG AAA AAC ATC-3'
E6 F	5'-GCG CGT TTC AAT TTG GCT TCT CTT T-3'
E6 R	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT AAG AAA ACT GTT CCA ATA C-3'
E7 R	5'-CGT CCC GCA ATA CTG GTA TGT ATT TAA C-3'
E7 R	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GGA TAT TTC TCC CAA TGA AAG-3'
E8 F	5'-CGG TTT CAC TTT TGG GTA AAT A-3'
E8 R	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC CCC CAC AAA ATG TTT AAT-3'
E9 F	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTC ACT AAA TAG TTT AAG ATG-3'
E9 R	5'-TTC ATT CTC TGG ATC AGA GT-3'

***GC-clamp portion of primer sequence is underlined**

Table 1.2 – Primer Sequences for LOH analysis.

LOH Marker	Primer Sequence	Dye
D10S1765 F	5'-ACA CTT ACA TAG TGC TTT CTG CG-3'	FAM
D10S1765 R	5'-CAG CCT CCC AAA GTT GC-3'	
AFMa086wg9 F	5'-AAA TGT ACG GTT CAT TGA CTT-3'	HEX
AFMa086wg9 R	5'-GAC TGA CTA CAA ATG GGC A-3'	
D10S541 F	5'-AAG CAA GTG AAG TCT TAG AAC CAC C-3'	FAM
D10S541 R	5'-CCA CAA GTA ACA GAA AGC CTG TCT C-3'	
D10S583 F	5'-TCT GAC CAA AAT ACC AAA AGA AC-3'	TET
D10S583 R	5'-AGA GAC TCC AGA TGT TTG ATG A-3'	

Table 1.3 – Primer sequences for MSI analysis.

MSI Marker	Primer Sequence	Dye
BAT25 F	5'-TCG CCT CCA AGA ATG TAA GT-3'	TET
BAT25 R	5'-TCT GCA TTT TAA CTA TGG CTC-3'	
BAT26 F	5'-TGA CTA CTT TTG ACT TCA GCC-3'	FAM
BAT26 R	5'-AAC CAT TCA ACA TTT TTA ACC C-3'	
D5S346 F	5'-ACT CAC TCT AGT GAT AAA TCG-3'	HEX
D5S346R	5'-AGC AGA TAA GAC AGT ATT ACT AGT T-3'	
D17S250F	5'-GGA AGA ATC AAA TAG ACA A-3'	TET
D17S250R	5'-GCT GGC CAT ATA TAT ATT TAA ACC-3'	
D2S123F	5'-AAA CAG GAT GCC TGC CTT TA-3'	HEX
D2S123R	5'-GGA CTT TCC ACC TAT GGG AC-3'	

Table 1.4 - Primer sequences and genotyping methodologies for the 10 SNPs.

SNP	Primer Sequence	Genotyping Method
SNP 1 F	CATTCTCAAGCAGGACTCAG	RFLP (*HincII*)
SNP 1 R	AATCCACCTGCTTCAGCTTC	
SNP 2 F	CCTGATGTTTAGAGAAGCAG	RFLP (*BfaI*)
SNP 2 R	CTTAGATTGCTGATCTTGTCTCC	
SNP 3 F	GGATCACAGATGTAGGCTTG	Fragment Analysis
SNP 3R	/FAM/-catcgccTAGCTGAGAGTGTACTAGAC	
SNP4 F	AGGAATTCATGTCTGATGTG	SNaPshot
SNP4 R	GTGACTGTACTGCTCACTTC	
	SBE primer: gtgcAATCAAATTTTTGTACCTACAA	
SNP5 F	TGTAACCTGCAGGAGGCATC	SNaPshot
SNP5 R	AAAGCAGAGAGGTAATACTC	
	SBE primer: attacgtaGACTACGACCCAGGTAGG	
SNP6 F	TGCTTGTTAGAGTGAGGTAG	RFLP (*NcoI)*
SNP6 R	CTAGCTCTATCAATCAGGTG	
SNP7 F	GCAACTGAATAGATGCGTAG	SNaPshot
SNP7 R	ATAACTAACACCATCGTCAC	
	SBE primer: cttaatccgtagtcaCCATTACTTCACCTCATCT	
SNP8 F	GGTACCAGGTACCAGATAAG	RFLP (*HpyCH4IV*)
SNP8 R	AAGGCAATCTGAGTTATCTG	
SNP9 F	ATTGCTTCGCTCACCTGCTC	RFLP (*HpyCH4IV*)
SNP9 R	CCTTTGAGATCCTCAGTAAG	
SNP10 F	TTGGCTACAAATGTCTCTAG	RFLP (*Bsu36I*)
SNP10 R	GGTGCTGCTGTTTACTGAG	

Table 2 – Characteristics of the 10 SNPs used in haplotype analysis

SNP	dbSNP ID	Position*	Variation (major/minor allele)	Location†
1	rs11202585	89,598,759	G/C	-15448
2	rs1903860	89,610,190	T/C	-4017
3	rs11355437	89,629,037	del/ins G	IVS1-14725
4	rs10887763	89,645,216	A/G	IVS2+1370
5	rs1234224	89,665,276	A/G	IVS2-9974
6	rs10490920	89,675,623	T/C	IVS3+329
7	rs2299941	89,694,699	A/G	IVS5-7156
8	rs52090929	89,710,231	T/C	IVS7-400
9	rs10509532	89,727,534	C/T	*12325
10	rs11202614	89,745,623	C/T	*304141

***SNP position on chromosome 10, March 2006 Human Genome assembly, NCBI Build 36.1, (hg18)**

†Location relative to translation start codon (-), PTEN introns (IVS), or translation stop codon (*)

Table 3 – Somatic *PTEN* mutations and their consequences.

***PTEN* Mutation**	***PTEN* Exon**	**Sample**	**Histology**	**FIGO Stage**	**Consequence**
TTA23GTA	1	AA12	Endometrioid	IIIC	L23V
IVS1+35C>T	1	AA6	Endometrioid	IC	splicing
IVS1+35C>T	1	NAA20	Mixed mullerian	IB	splicing
GGA36GAA	2	AA12	Endometrioid	IIIC	G36E
CCT38TCT	2	NAA5	Endometrioid	IIB	P38S
156insA	2	AA12	Endometrioid	IIIC	frameshift
IVS4-30~38delT	5	AA13	Mixed epithelial	IIIC	splicing
IVS4-30~38delT	5	NAA15	Mixed mullerian	IB	splicing
CCA96CGA	5	NAA3	Endometrioid	IC	P96R
CTA112GTA	5	AA8	Endometrioid	IB	L112V
TGT124TAT	5	AA10	Endometrioid	IA	C124Y
GGA127GAA	5	NAA14	Mixed epithelial	IIIC	G127E
CGA130GGA	5	NAA5	Endometrioid	IIB	R130G
CGA130TGA	5	AA13	Mixed epithelial	IIIC	R130X; truncating
CGA130TGA	5	NAA11	Endometrioid	IB	R130X; truncating
CGA130GGA	5	NAA14	Mixed epithelial	IIIC	R130G
CGA130GGA	5	NAA15	Mixed mullerian	IB	R130G
IVS5+1~5del5	5	AA5	Endometrioid	IIB	splicing
610~611delCC	6	NAA15	Mixed mullerian	IB	frameshift
CGA233TGA	7	AA7	Endometrioid	IB	R233X; truncating
CGA233TGA	7	AA11	Endometrioid	IB	R233X; truncating
795~800delA	7	AA10	Endometrioid	IA	frameshift
956~959delCTTT	8	AA5	Endometrioid	IIB	frameshift
969insA	8	AA13	Mixed epithelial	IIIC	frameshift
TAC336TAG	8	NAA6	Endometrioid	IC	Y336X; truncating

Table 4.1 – *PTEN* mutation, LOH, and MSI in relation to race, histology, and FIGO tumor stage.

	***PTEN* Mutation**		**LOH**		**MSI**	
Race	**Freq.**	***P***	**Freq.**	***P***	**Freq.**	***P***
AA	8/26 (31%)	0.766	15/25 (60%)	0.382	5/25 (20%)	1.000
NAA	7/27 (26%)		19/26 (73%)		5/26 (19%)	
Histology	**Freq.**	***P***	**Freq.**	***P***	**Freq.**	***P***
Endometriod	11/24 (46%)	0.015*	15/23 (60%)	1.000	4/23 (15%)	1.000
Other	4/29 (14%)		19/28 (87%)		6/28 (21%)	
FIGO Stage	**Freq.**	***P***	**Freq.**	***P***	**Freq.**	***P***
IA or IB	7/31 (23%)	0.357	20/29 (69%)	0.769	4/29 (14%)	0.295
IC or higher	8/22 (36%)		14/22 (64%)		6/22 (27%)	

***When analyzed separately, this association was statistically significant in the AA group (*P* = 0.009), but not in the NAA group (*P* = 0.662).**

Table 4.2 – *PTEN* Mutation, MSI, and LOH status compared to each other.

	LOH		**MSI**	
***PTEN* Mutation**	**Freq.**	***P***	**Freq.**	***P***
Yes	13/15 (87%)	0.060	5/15 (33%)	0.135
No	21/36 (58%)		5/31 (16%)	
LOH			**Freq.**	***P***
Yes			9/34 (26%)	0.135
No			1/17 (6%)	

Table 5.1 – Pearson χ^2 of Reconstructured Haplotypes for AA and NAA

		Race		Histology		FIGO Stage	
	Extended Haplotypes	**AA n = 52**	**NAA n = 54**	**Endo. n = 48**	**Other n = 58**	**IA/IB n = 60**	**>IC n = 46**
1	CTIAATATCC	18	13	15	16	22	9
2	CTDAATACCC	3	9	9	3	7	5
3	CTIAGTATCC	19	8	10	17	12	15
4	GCIGGCGTTT	3	8	7	4	5	6
5	CTDAATATCC	2	6	3	5	6	2
6	GTIAGTATCC	2	5	3	4	4	3
7	CTIAATATCT	5	5	1	9	4	6
8	GCIGGCATTT						
9	CTIAATATTC						
10	GCIGGCATTC						
11	GTIAGCATCC						
12	GTIAGCATTT						
13	GCIAGTATCT						
		χ^2 = 13.814, *P* = 0.032		**χ^2 = 11.870 *P* = 0.065**		**χ^2 = 7.026 *P* = 0.318**	

		***PTEN* Mutation**		**LOH**		**MSI**	
	Extended Haplotypes	**Yes n = 30**	**No n = 74**	**Yes n = 68**	**No n = 34**	**Yes n = 20**	**No n = 82**
1	CTIAATATCC	7	24	22	9	5	26
2	CTDAATACCC	4	8	8	4	2	10
3	CTIAGTATCC	8	18	14	12	4	22
4	GCIGGCGTTT	6	5	7	4	2	9
5	CTDAATATCC	1	7	6	0	3	3
6	GTIAGTATCC	3	4	5	2	1	6
7	CTIAATATCT	1	8	6	3	3	6
8	GCIGGCATTT						
9	CTIAATATTC						
10	GCIGGCATTC						
11	GTIAGCATCC						
12	GTIAGCATTT						
13	GCIAGTATCT						
		χ^2 = 7.387 *P* = 0.287		**χ^2 = 5.298 *P* = 0.506**		**χ^2 = 5.329 *P* = 0.502**	

Table 5.2 – *P*-values for Reconstructed Haplotypes

	Race			Histology			FIGO Stage		
Hap	**AA n = 52 (%)**	**NAA n = 54 (%)**	***P***	**Endo. n = 48 (%)**	**Other n = 58 (%)**	***P***	**IA/IB n = 60 (%)**	**>IC n = 46 (%)**	***P***
1	18 (35%)	13 (24%)	0.287	16 (32%)	15 (27%)	0.520	22 (37%)	9 (20%)	0.084
2	3 (6%)	9 (17%)	0.124	9 (18%)	3 (5%)	0.034	7 (12%)	5 (11%)	1.000
3	19 (37%)	8 (15%)	0.014	10 (20%)	17 (30%)	0.375	12 (20%)	15 (33%)	0.178
4	3 (6%)	8 (15%)	0.202	7 (14%)	4 (7%)	0.219	5 (8%)	6 (13%)	0.526
5	2 (4%)	6 (11%)	0.271	3 (6%)	5 (9%)	0.726	6 (10%)	2 (4%)	0.461
6	2 (4%)	5 (9%)	0.438	3 (6%)	4 (7%)	1.000	4 (7%)	3 (7%)	1.000
7	0 (0%)	2 (4%)	0.495	0 (0%)	2 (4%)	0.500	0 (0%)	2 (4%)	0.186
8	2 (4%)	1 (2%)	0.614	1 (2%)	2 (4%)	1.000	2 (3%)	1 (2%)	1.000
9	0 (0%)	1 (2%)	1.000	0 (0%)	1 (2%)	1.000	1 (2%)	0 (0%)	1.000
10	0 (0%)	1 (2%)	1.000	0 (0%)	1 (2%)	1.000	0 (0%)	1 (2%)	0.433
11	1 (2%)	0 (0%)	0.491	0 (0%)	1 (2%)	1.000	0 (0%)	1 (2%)	0.433
12	1 (2%)	0 (0%)	0.491	1 (2%)	0 (0%)	0.453	0 (0%)	1 (2%)	0.433
13	1 (2%)	0 (0%)	0.491	0 (0%)	1 (2%)	1.000	1 (2%)	0 (0%)	1.000

Further Analysis of Haplotype 3

	***PTEN* Mutation**			**LOH**			**MSI**		
Hap	**Yes**	**No**	***P***	**Yes**	**No**	***P***	**Yes**	**No**	***P***
3	8/30 (27%)	19/76 (25%)	1.000	14/68 (21%)	12/34 (35%)	0.148	4/20 (20%)	22/82 (27%)	0.775

FIGURE LEGENDS

Figure 1

Schematic diagram of the *PTEN* locus with the 4 LOH markers and 10 SNPs included in the current analysis.

Figure 2

LOH status is depicted at each marker (D10S1765, AFMa086wg9, D10S541, and D10S583) for all AA and NAA samples. For each sample, overall LOH was scored when one or more of the panel of four polymorphic loci showed LOH, as is standard.

Figure 3

MSI status is provided for each marker (BAT25, BAT26, D2S123, D5S346, and D17S250) for all AA and NAA samples. Samples were defined as MSI-positive when at least 60% (3 out of 5) of the markers displayed instability. A sample was classified as MSI-negative when it did not meet this criterion.

Figure 4

GOLD plots of pairwise LD between the 10 SNPs in the AA and NAA study groups. *D'* values are reported for both racial groups. Excluding unique areas of moderate/low LD, the data show that the *PTEN* locus is primarily characterized by strong LD (*D'* = 1.00).

FIGURES

Figure 1

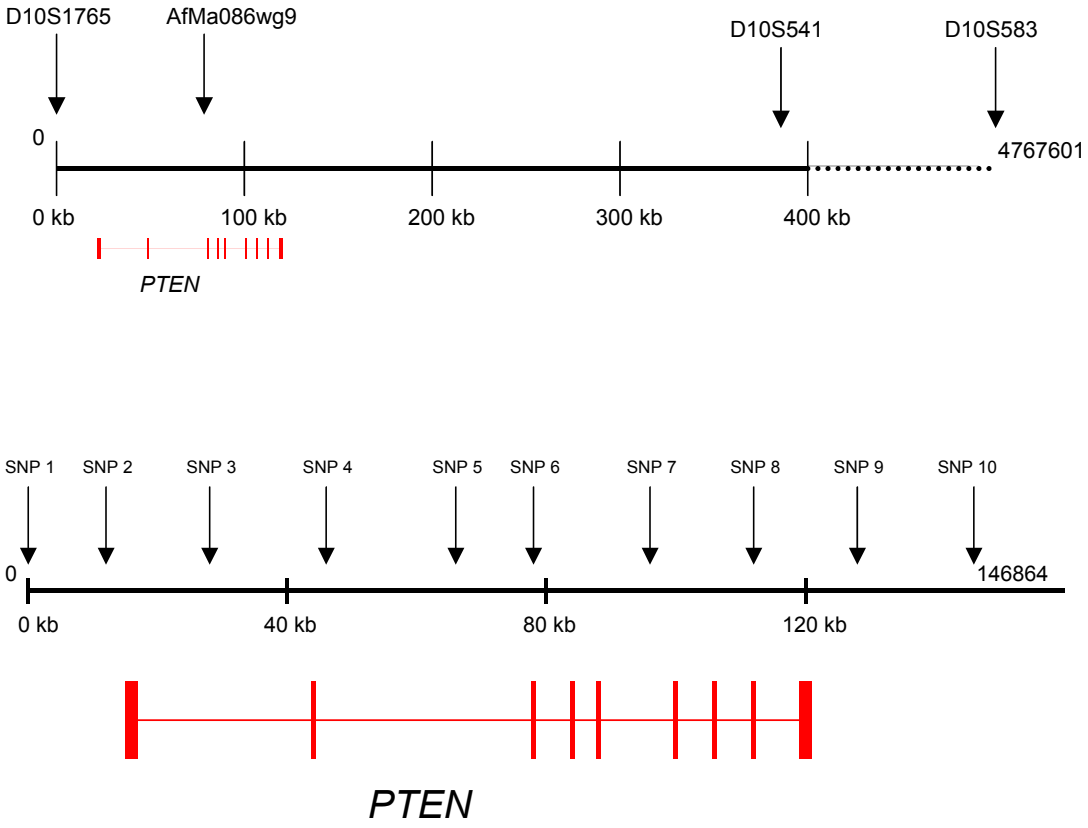


Figure 2

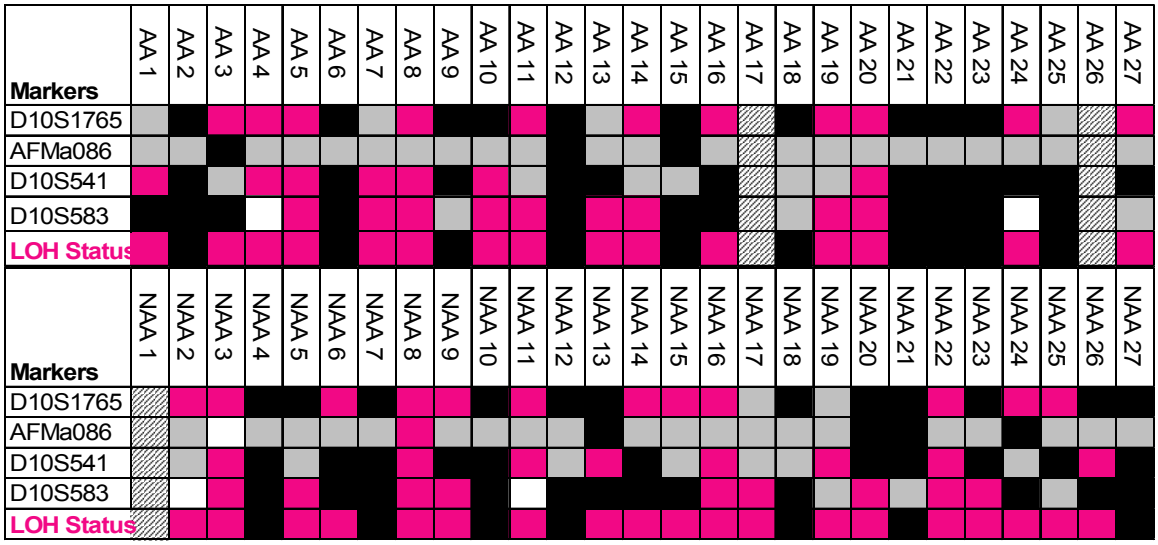


Figure 3

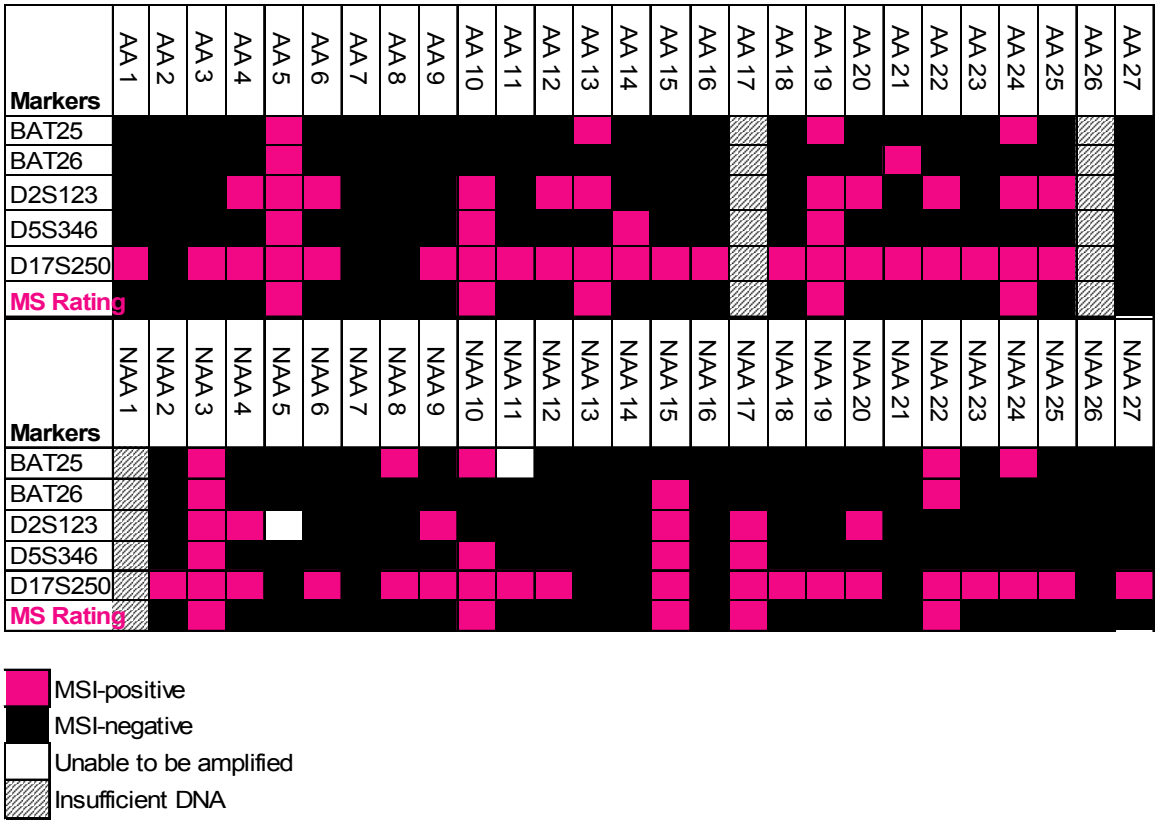
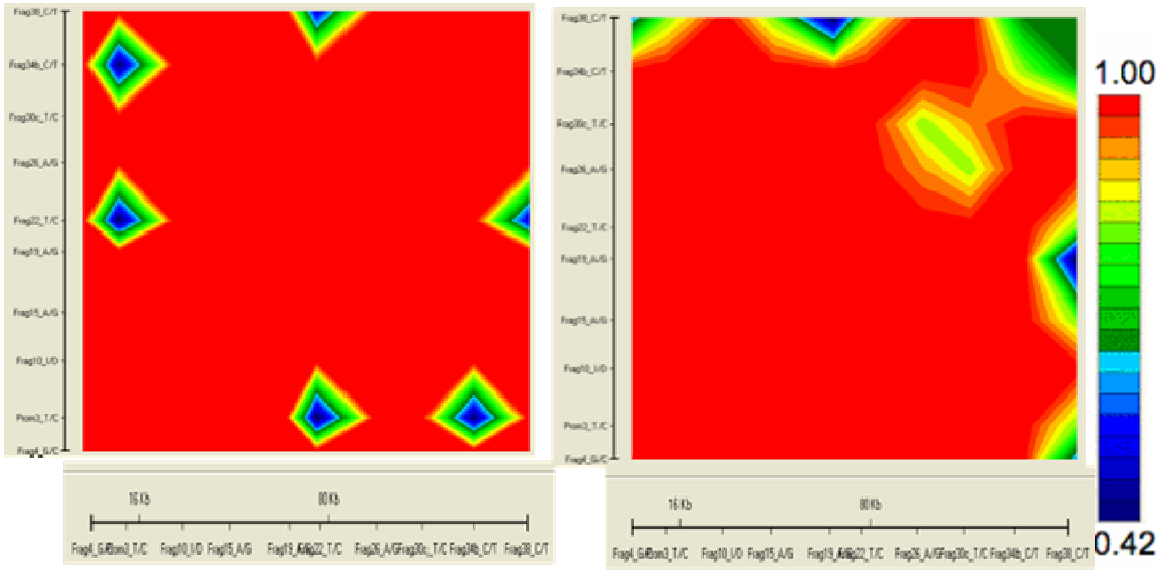


Figure 4



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